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MicroRNA 7 downregulates alpha-synuclein expression: An in vivo study of an early intervention strategy

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Abstract
Aim: In this study, we aimed to investigate in vivo the modulation of striatal alpha-synuclein (α-SYN) in the rotenone model of PD as an early intervention strategy using miRNA (miR-7) before the establishment of pathology.
Material and Methods: Parkinson's disease was induced in male Wistar rats by subcutaneous injection of rotenone (2.5mg/kg) daily for 5 weeks. Rats received a single bilateral striatal injection of miR-7 mimic 2h before injection of rotenone. The expression of striatal miR7, α-SYN mRNA, α-SYN protein were assessed.
Results: Early intervention by miR-7 mimic succeeded to elevate the striatal level of miR7and to decrease α-SYN overexpression in vivo.
Discussion: The early intervention with miR-7 mimic could reverse pathology and downregulates α SYN overexpression in rotenone-induced rat model at the transcriptional and post- transcriptional levels.

Keywords
Parkinson's Disease, α-SYN Gene Overexpression, Chitosan Nanoparticles, miRNA7 Mimic

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Introduction

Parkinson's disease (PD) is a neurodegenerative disease affecting 1% of those over the age of 60 [1]. Clinically, PD is characterized by motor symptoms of rest tremor, bradykinesia (or akinesia), and postural instability, and non-motor symptoms such as olfactory dysfunction, constipation, and cognitive impairment [1,2]. The association of aging with PD is well detailed, and aging is documented to be the most important risk factor for the development of PD [1]. The prevalence of PD and the burden of sick people will increase in future due to an increase in the elderly population [3]. One of the main mechanisms of Parkinson's disease pathophysiology is the overexpression of the α-synuclein (α-SYN) protein and the subsequent formation of Lewy bodies, which are small acidic protein enriched in the presynaptic terminals of neurons [4]. The fact that α-SYN protein overexpression is implicated in cognitive and motor impairments in PD has attracted many researchers as a new potential therapy for PD [5]. The miRNAs are capable of modulating several signaling pathways in several diseases such as Parkinson's disease, making them potent modulators of gene expression [6]. Based on previous studies, normal level of miR-7 permits normal neurogenesis in the central nervous system, also keeps α-SYN protein expression at the physiological level [7]. Furthermore, the role of miR-7 on α-SYN protein downregulation, at both the transcript and protein level was documented by Doxakis and colleagues [8]. Herein, we use miR-7 mimic in vivo to investigate its role as an early intervention strategy for PD. Chitosan nanoparticles were chosen as a delivery system for our treatments, whereas many studies documented its unique ability for cell transfection in vivo and in vitro [9]. To date, the implication of miR-7 in vivo as an early intervention in parkinsonism remains unclear.

Material and Methods

1. Preparation of nanoparticles

1.1 Preparation of chitosan nanoparticles

Chitosan nanoparticles were prepared by the ionic gelation method following Zhao and Wu method [10]. The formation of chitosan nanoparticles was shown by the turbidity of the solution. Next, the nanoparticles were collected by centrifugation, freeze-dried (Buchi, Lyovapor L-200, Switzerland) and stored at 4 ºC for characterizations.

Concerning FITC-labeled chitosan nanoparticles, 5-10 mg of the FITC dye was dissolved in TPP solution and added dropwise to the chitosan solution under magnetic stirring at room temperature in the dark. FITC-labeled chitosan nanoparticles were centrifugated and washed with DIH2O till the free FITC could not be detected in the supernatant.

1.2. Preparation of chitosan/miRNA nanoplexes

CS/miR-7 mimic nanoplexes were prepared via the ionic gelation method. Accordingly, lyophilized miRNA 7 (Qiagen. Cat. No. 219600) was dissolved in RNAase- free buffer solution and was added by pipetting to chitosan solution (20µg /ml). The mixture was then vortexed and incubated for 45 min with vigorous shaking for 30 min at room temperature till the formation of nanoplexes [11].

1.3. Nanoparticles Characterization

Particle size, surface charge of freshly prepared CS NPs and chitosan /miRNA samples were measured using a Zetasizer Nano ZS (Malvern Instruments, UK), based on Photon Correlation Spectroscopy (PCS) techniques. No dilutions were performed during the analysis. The measurements were made at 25ºC [12].

1.4. Morphological Analysis

Morphological characterization of unloaded CS NPs, miR-7 loaded CS NPs was carried out using JEM-1400 series 120 kv transmission electron microscopy (TEM), USA [12].

1.5. The loading efficiency of miRNA

Onto chitosan NPs was obtained by measuring the total miRNA concentration added, compared with supernatant free miRNA concentration after loading to CS NPs [13].

2. In vivo study

2.1 Laboratory animals

Adult male albino rats (300-350 g, 14 weeks) were obtained from the Physiology Department Animal House, Alexandria Faculty of Medicine, Egypt. They were maintained on standard conditions (natural dark/light cycle, controlled room temperature (25±2oC), with free access to water and food. The ethical guidelines of Alexandria University on laboratory animals and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 80-23, revised 1978) were adopted. Further, the Ethics Committee of Alexandria Faculty of Medicine approved this study.

2.2 Study design

The study was conducted on 33 rats divided into 3 groups:

- Control group (11 rats) received a bilateral striatal injection of a single dose (1µl) of phosphate-buffered saline (PBS) using a stereotaxis technique 2 hours before S.C injection of DMSO, daily for 5 weeks (negative control group). Disease group (11 rats): received a bilateral striatal injection of a single dose (1 µl) of PBS using stereotaxis technique 2 hours before s.c injection of 1ml of Rotenone (2.5mg/kg) [14], dissolved in DMSO) daily for 5 weeks (positive control group). Treated group (11 rats): received bilateral striatal injection of a single dose (1µl) of chitosan (Sigma Aldrich, USA) nanoparticles incorporated with miR-7 mimic (50 pmole/µl/site of injection) [15] using stereotaxis technique 2 hours before s.c injection of 1ml of Rotenone dissolved in DMSO (2.5mg/kg) daily for 5 weeks as illustrated in Figure 1.

3. Animals sacrifice and Striatal tissue sampling

The animals were sacrificed using ether anesthesia after the end of 5 weeks. Then whole brains were removed, rinsed with ice-cold saline and each striatum was immediately dissected and stored at -80ºC for biochemical analysis.

3.3. Quantitative reverse transcription PCR (qRT PCR) for miRNA 7

Striatal total RNA, including miRNA, was extracted following the manufacturer's protocol of miRNeasy Mini Kit (Qiagen, Hilden, Germany). Then, TaqMan miRNA reverse transcription was carried out with specific miRNA 7 primers (Applied Biosystems, USA) . The TaqMan microRNA assay system was then used for quantification of miRNA 7 using the TaqMan® Universal PCR Master Mix II (Applied Biosystems, USA). The amplification reaction was run in StepOne real-time PCR system (Applied Biosystems, USA) and StepOne™ Software v2.3 was used for data analysis using the 2-ΔΔCt method for calculation of gene

expression relative to the housekeeping U6 snRNA [16].

3.4. Quantitative reverse transcription PCR (qRT PCR) for α-SYN

Reverse transcription was done following the protocol of reverse transcriptase (RT) Superscript II kit (Invitrogen, USA). The amplification of α-syn cDNA was performed in StepOne real-time PCR system (Applied Biosystems, USA). Then, the expression of α-syn mRNA was calculated using the comparative CT method relative to the housekeeping gene GAPDH.[16]

3.5 Western Blot analysis of α-SYN protein

Striatal tissue samples were homogenized in radioimmunoprecipitation (RIPA) buffer Next, BCA protein assay was adopted to measure the lysate total protein concentration. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotting to nitrocellulose membranes (Bio-Rad, Mississauga-Canada) were done. The membranes were then incubated with the specific primary antibodies and corresponding secondary antibody and protein bands were visualized using 3,3',5,5'-tetramethylbenzidine (TMB) stain. Quantification of protein bands was done using Quantity One software (Bio-Rad Laboratories-USA), where protein expression was normalized to the control group and β-actin protein [17]

Statistical analysis

Data were expressed as median and interquartile range. Statistical analysis was performed with IBM SPSS statistics, version 21.0 (IBM Inc.). The results were analyzed using the Kruskal-Wallis test, Pairwise comparison between each 2 groups was done using the Post Hoc Test (Dunn's for multiple comparisons test). P-value ≤0.05 was defined to be statistically significant

Results

1. Zeta size and Zeta potential of nanoplexes

Zeta sizer's results documented that the miRNA 7 loaded nanoparticles measured about 200 nm. The PDI value of chitosan nanoparticles was miRNA 7 loaded chitosan nanoparticles was 0.62, thus indicating a narrow and acceptable particle size distribution (PDI <0.7) (Figure 2 A). Zeta potential of miRNA-loaded chitosan nanoparticles revealed the positivity of nanoplexes, which is favorable for good cellular uptake (Figure 2 B). These results showed that the fabricated nanoplexes are of good size and potential.

2. Morphology

Chitosan nanoparticles and chitosan-loaded miRNA 7 mimic nanoparticles morphology was analyzed by TEM. In the present study, TEM images revealed that chitosan nanoparticles have a nearly spherical shape, regular surface, and different sizes ranging from 120 to 200 nm on scale bar 200 nm, while the chitosan loaded miRNA 7 nanoparticles show semi- spherical shape, irregular and rough surface with a size range from 120 to 200 nm. These results showed (Figure 2 C&D) a slight change in size between the chitosan nanoparticles and the loaded nanoplexes. In addition, surface irregularity of the nanoplexes may be explained by the success of miRNA loading.

3. The loading efficiency of miRNA

The efficiency of miRNA encapsulation inside chitosan nanoparticles was measured using a UV spectrophotometer. Data analysis via nanometer revealed 95% loading efficiency

Figure 1. Schematic illustration of the study design and timeline of the preventive experiment created by Biorender.com (72x72 DPI)

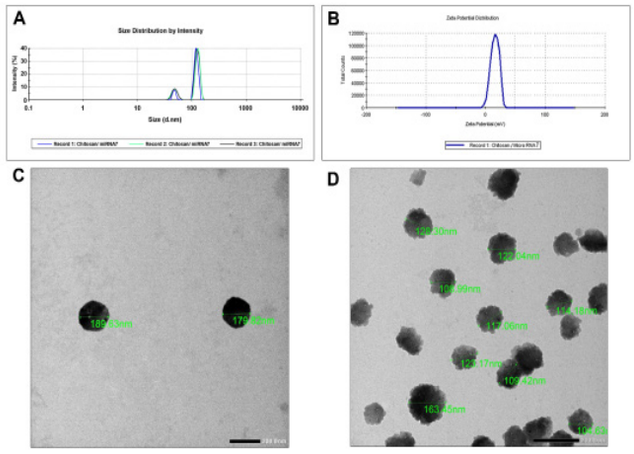

Figure 2. Characterization of nanoparticles according to their size and morphology, A. Zeta size of CS/ miR-7 NPs, B. Zeta potential of CS/miR-7 NPs, C&D. morphology of chitosan nanoparticles, CS/miR -7 nanoplexes using TEM 120 kv, scale bar, 100 nm: C. Unloaded CS NPs showing a smooth surface, D. CS/miR-7 nanoplexes showing a rough surface

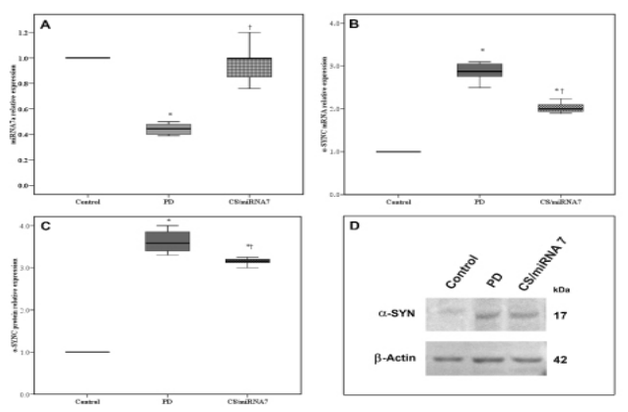

Figure 3. Effect of striatal administration of miR-7; A: Striatal miRNA 7 expression, B: Striatal α-SYN mRNA expression, C&D: Striatal α-SYN protein expression; C Western blot results were quantified relative to the control group to be expressed in arbitrary units, D Representative immunoblot demonstrating the expression of brain α-SYC protein and β-actin that was probed as an internal control. *P≤0.05, versus control group, †P≤0.05, versus PD group.

of miRNA incorporated inside chitosan nanoparticles.

4. Expression of striatal miR-7

There was a statistically significant decrease in the Striatal miR-7 expression level of the rotenone group (PD group) as related to the control group. The miR-7 mimic treatment succeeded to increase striatal miRNA 7level, which was almost near to the control group (Figure 3A).

5. Effect of miR-7 administration on striatal α-SYN mRNA expression

There was a statistically significant increase in the Striatal level α-SYNC mRNA expression in the rotenone group (PD) as related to the control group. Treatment with miR-7 mimic succeeded to decrease striatal α-SYNC mRNA expression levels significantly in relation to the rotenone group but still, there was significant difference with the control group (Figure 3B).

6. Effect of miR-7 administration on striatal α-SYN protein expression

The present study showed that there was a statistically significant increased striatal α-SYNC protein expression in the rotenone group (PD) versus the control group. Early intervention with miR-7 mimic succeeded to decrease protein expression compared to the rotenone group, while failed to normalize it (Figure 3C&D).

Discussion

To date, this is the first research that investigated miRNA 7 as early intervention strategy in vivo for Parkinson's disease and studied its potential role in different aspects of the disease. A variety of nanoparticles have been fabricated for the delivery of miRNA therapies. In the current study, we selected chitosan as miRNA delivery platform. It is well known that chitosan is a Cationic polymer, which can form complexes with negatively charged materials due to its abundant amine groups thus chitosan is a promising delivery system [18]. The obtained particle morphology and size are in line with previous studies [12]. Concerning the success of miRNA loading , the present study reported 95% of loading efficacy, which is in parallel to previous reports, which have documented a high loading efficacy between chitosan nanoparticles and negatively charged materials as miRNAs [13, 18].

miR-7 is expressed in different areas of the brain and binds selectively to the 3'UTR of the α- SYN gene, which significantly decreases the synthesis of α-SYN and inhibits its aggregation [19]. Time of intervention is a cornerstone in stopping the progression of the disease [20], thus we investigated its early administration. Initially, we found that miR-7 was down expressed in PD rats, and its expression, following the treatment strongly, restored its normal level, loss of dopaminergic neurons and downregulated the overexpression of α-SYN protein in PD rats. Taken together, these results confirm that miR-7 could target α-SYN overexpression to attenuate its pathological sequence in PD when injected early. It is well known that dopamine depletion and α-SYN accumulation are the major diagnostic features of PD [21]. Previous study has shown loss of normal striatal level of miR-7 as pathological features of PD [22]. In accordance with these previous results, our study revealed reduced level of miR-7 in striatal tissues in rotenone-induced PD rats. Early intervention with miR-7 mimic succeeded to increase its striatal expression and restored its level to normal. Similarly, Zhou et al reported the restoration of miRNA 7 in the MPTP mice model upon administration of the miRNA 7 mimic [23]. Interestingly, peripheral administration of rotenone-induced α-synuclein aggregation with the formation of Lewy bodies, similar to those observed in PD [14]. The same observations were detected in the current study, as we reported an increase in α- synuclein mRNA relative expression and α- synuclein protein expression in striatal tissues of rotenone induced PD rats. In the present study, α-synuclein mRNA and protein expression was efficiently modulated by early intrastriatal miR-7 mimic injection. The direct effect of miR-7 on α-synuclein expression was first documented, in vitro, by Junn et al who demonstrated that transfection with 40 nM of miR-7 in HEK293T cells resulted in a decline of α-synuclein expression both at protein and mRNA levels [7]. mTOR pathway and autophagy are among the mechanisms that mediate the regulation of α- synuclein expression by miR-7 as reported in vitro [24]. Furthermore, a pervious study declared that miR-7 downregulates α-SYN expression by targeting mRNA, causing prevention of α-synuclein protein expression [25]. Consequently, these promising results particularly in relation with early intervention strategy before establishing pathology augments the neuroprotective role of miR-7 as an effective modifying strategy in PD

Conclusion

In conclusion, the results of our study offer insights into early intervention with miR-7 in rats with PD; upregulation of miR 7 expression prevented α- SYN aggregation Moreover, these data give clue to therapeutic potential for miR7 early in the treatment of PD, which requires a broadening of the therapeutic perspective through the usage of frequent doses and different duration of treatment.

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Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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